cardiomyopathy (AC). AC is characterized by fibrofatty replacement of myocardium, which can lead to fatal ventricular arrhythmias. Titin is a gigantic sarcomeric protein that is responsible for passive tension development in stretched cardiac muscle. We investigated the effect that the AC-linked point mutation (T16I in Ig10) has on the mechanical and kinetic stability of Ig10 using single molecule force spectroscopy (AFM) and degradation assays. Our AFM data indicate that T16I Ig10 unfolds at a lower force than WT Ig10, which shows that it is mechanically less stable. We also performed force clamp and refolding experiments with AFM and found that the unfolding rate of T16I Ig10 is four times higher than WT Ig10 (.02/s vs. .005/s at zero force), but that this difference is further increased in a force-dependent manner; refolding rates were found to be similar. We also performed degradation assays with the protease tryspin to determine if the T16I mutation affects the highly-stable beta barrel tertiary structure typical of Ig domains. We found that T16I Ig10 has a greater proteolytic susceptibility compared to WT Ig10, and subsequent mass spectrometry analysis determined that the primary cleavage site in T16I Ig10 is the lysine residue that flanks T16I (K17). This strongly suggests that the disease-linked titin mutation disrupts the local structure of Ig10 in the vicinity of T16, which leaves Ig10 vulnerable to cleavage. Peptide bond breakage anywhere along titin's elastic region (which includes Ig10) would likely abolish titin's ability to generate force and lead to severe physiological responses.

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Effect of Shortening Titin's Proximal IG Segment on Passive Cellular Mechanics

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Titin is widely considered the main contributor to passive stiffness of cardiac myocytes. To test this we studied a novel mouse model, in which a large fraction of titin's proximal tandem Ig segment is deleted (we refer to the model as IG KO; for details on model, see Chung et al poster). The proximal tandem Ig segment extends greatly under physiological conditions and shortening the tandem Ig segment is predicted to increase titin-based passive tension. We first studied passive tension in skinned cardiac myocytes by stretching cells in relaxing solution with a velocity of 1 base-length/s (~*in vivo* speed during diastole), and determining stiffness from the slope of the passive stress - SL relation in the physiological SL range of 1.95-2.10 μm. Cellular stiffness was significantly increased in the IG KO by ~50%. Using a sinusoidal analysis, the elastic moduli at physiological SLs were found to be increased in the IG KO but the viscous moduli were not different. This shows that the tandem Ig segment is a pure elastic spring. Currently we are investigating intact myocytes using a carbon-fiber based system for attaching cells and measuring force. We conclude that the proximal Ig segment of titin's I band region is a pure elastic spring and that shortening of this spring element increases titin-based cellular stiffness in the physiological SL range. Increased titin-based stiffness is found in patients with heart failure with preserved ejection fraction (HFpEF) and the IGKO is well-suited to study the effect of increased titin-based stiffness on heart function (see poster by Chung et al).

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The Multifunctional Calcium/Calmodulin-Dependent Protein Kinase II Delta (CaMKIIδ) Phosphorylates Titin N2B and PEVK Spring Elements

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Titin is phosphorylated by several kinases that significantly affect titin mechanical properties. PKA and PKG phosphorylates titin N2B element and decreases titin's stiffness, while PKCα phosphorylates titin PEVK spring element and increases titin's stiffness. CaMKII is a Ca^{2+} and calmodulin dependent serine/threonine kinase that is activated by increases in cellular Ca^{2+}. Four isoforms have been described (α, β, γ, and δ). CaMKII delta (CaMKIIδ) is the predominant isoform in the heart. It has been found that increases in CaMKIIδ activity causes severe left ventricular dysfunction. The aim of the present study is to determine whether CaMKIIδ phosphorylates titin and, if so, identify its target domains. To determine whether CaMKIIδ phosphorylates titin in mouse left ventricular muscle, LV skinned fibers were incubated with recombinant human CaMKIIδ expressed in insect cells and [γ-^{32}P]ATP. We found that CaMKIIδ phosphorylates titin in mouse LV skinned fibers. Further, pre-incubation of the LV skinned fiber with protein phosphatase 1 (PP1) significantly increases ^{32}P incorporation on titin. To identify the CaMKIIδ titin target domains, human titin N2B, PEVK, Ig8-15, I27-34, and murine N2B recombinant proteins expressed in *E. coli* were incubated with CaMKIIδ and [γ-^{32}P]ATP. We found that titin N2B and PEVK spring elements, but not Ig domains, were phosphorylated by CaMKIIδ. Prediction of the specific phosphorylation sites suggest overlap with the PKC and PKA sites (including S170 of the PEVK element a PKC substrate) and work to indentify the specific substrate sites and the possible effect on mechanics is in progress. In summary, the present study showed that CaMKIIδ phosphorylates titin spring elements.

2841-Pos Board B611

Hypothyroidism Effects on Wild Type and Mutant Rats with Altered Cardiac Titin Expression

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Thyroid hormone levels play an important role in cardiac regulation, and a state of hypothyroidism leads to dilated forms of cardiomypathy. Inappropriate thyroid levels can also impact the sarcomeric protein titin, which is responsible for maintaining passive tension and structural integrity. The effects of hypothyroidism were studied after administration of propylthiouracil (PTU) to wild type (Wt) and homozygous mutant (Hm) rats (Greaser et al J Mol Cellul Cardiol 44:983, 2008) (the latter of which express a giant titin isoform). Enzyme-linked immunosorbent assay results demonstrated that PTU lowered thyroxine levels in the treated rats. Electrophoretic cardiac myosin heavy chain analysis indicated that hypothyroidism induced a transition to predominantly beta myosin heavy chain expression, while Wt control samples continued to express an age-appropriate range of alpha and beta isoforms. PTU-treated Wt rats had a larger N2BA to N2B titin ratio, and the slower migrating N2BA was in higher proportion than the faster N2BA. PTU did not affect titin isoform expression in Hm. Because it is believed that thyroid hormone regulates titin isoform expression through the PI3K/Akt pathway, we investigated several participants by immunoblotting. The Akt PH domain was not altered by PTU administration, although expression was higher in Hm rats. Akt phospho-Ser473 did not vary between treatments, but mTOR phospho-Ser2448 demonstrated a higher phosphorylation state in Wt. Echocardiography data showed significantly decreased fractional shortening in Hm control, Hm PTU, and Wt PTU relative to Wt control. Wt control also exhibited a higher ejection fraction and a lower isovolumic relaxation time compared to the other treatments. It can be concluded that hypothyroidism has significant effects on titin isoform expression and other cardiac functional characteristics. Supported by NIH HL77196.

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Characterization of Myocardial Passive Stiffness in a Mouse Model of Volume Overload Heart Failure

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ABSTRACT

Volume overload (VO) heart failure occurs due to pathologies such as mitral valve regurgitation, infarcts and ventricular septal defects, and leads to characteristic eccentric dilation. Changes in passive myocardial stiffness are not well understood and here we dissected the contribution of extracellular matrix (ECM)-based and titin-based stiffness to overall passive stiffness. Titin is a giant protein that regulates passive tension and hence diastolic function in the myocardium. In heart failure, differential splicing and phosphorylation events can occur that alter the stiffness of this protein and influence hemodynamics. Increased diastolic stiffness due to differential splicing and phosphorylation of titin has been observed in pressure overload hypertrophy; however, there are no published studies investigating whether pure LV VO during compensated heart failure leads to changes in titin based passive stiffness. We studied the role of titin in modulating diastolic function in VO induced by aortocaval fistula (ACF) in the mouse. ACF was induced in three-month-old male C57BL/6 animals and allowed to progress for 12 weeks. At 12 weeks, echocardiography confirmed the presence of eccentric dilation and decreased systolic function as expected. Tissues were obtained from control and VO hearts for mechanical measurements and protein studies. Skinned muscle mechanics indicated an increase in both titin and ECM based passive based stiffness; we are currently investigating the molecular basis of the changes in titin. In summary, VO causes an increase in passive stiffness to which titin contributes.